p53 Protein Overexpression in Bone Marrow Biopsies of Patients With Shwachman-Diamond Syndrome Has a Prevalence Similar to That of Patients With Refractory Anemia

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● ***Context.*—Shwachman-Diamond syndrome (SDS) is a rare inherited disorder characterized by pancreatic insufficiency, neutropenia, and in some patients, metaphyseal dysostosis. Patients with SDS are at a high risk for development of bone marrow failure, myelodysplastic syndrome, and acute leukemia. The *p53* gene plays a major role in cell-cycle regulation, particularly in the presence of a genetic alteration in DNA, a critical step for the initiation of leukemogenesis. *p53* gene up-regulation and p53 protein overexpression may occur as a cellular reaction to significant DNA damage. Shwachman-Diamond syndrome and refractory anemia patients have close similarities in the prevalence of acute leukemia and in cell-cycle changes in bone marrow cells. This similarity was further investigated for p53 protein overexpression using archived tissue from patients with hematologic diseases having various leukemic propensities, including SDS and refractory anemia.**

***Methods.*—Immunohistochemical staining for p53 protein overexpression was performed on bone marrow biopsies from 9 patients with SDS. These specimens were compared with biopsies from 71 patients with acquired hematologic disorders with variable risk levels for leukemia, including acquired aplastic anemia (n = 14), refractory anemia (n = 46), and various acquired cytopenias (n = 11), as well as 37 control subjects.**

***Results.*—p53 protein overexpression was identified only in patients with SDS and in patients with refractory anemia; these groups exhibited comparable prevalences of 78% and 72%, respectively. None of the patients with acquired aplastic anemia, acquired cytopenias, or in the control group showed overexpression of p53 protein.**

***Conclusion.*—The prevalence of p53 protein overexpression in SDS is significantly different from that in acquired aplastic anemia and acquired cytopenias, but it is similar to the prevalence in refractory anemia. We speculate that p53 protein overexpression in this bone marrow failure syndrome may represent an early indicator of significant DNA genetic alteration, which is a crucial step in the process of leukemogenesis.**

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Shwachman-Diamond syndrome (SDS) is a rare inherited disorder characterized by pancreatic insufficiency and neutropenia, as well as metaphyseal dysostosis in slightly less than half the cases.[1,2] More than 200 patients have been identified with SDS.[2] Patients with SDS are at a high risk for development of bone marrow (BM) failure, myelodysplastic syndrome, and acute leukemia, and more than 10% of SDS patients develop myelodysplastic syndrome and/or acute leukemia. Published reports indicate that the risk for development of acute leukemia in refractory anemia (RA) is approximately 10%, which closely matches that of patients with SDS.[2–4] Moreover, a recent study of BM mononuclear cells in SDS reported a high expression of Fas antigen and a high prevalence of apoptosis. The authors observed similarity between SDS and myelodysplastic syndrome in cell-cycle characteristics.[5] To further investigate the similarities between SDS and myelodysplastic syndrome, we studied an important regulator of the cell cycle, p53 protein. Our rationale is based on the fact that the multistep process of leukemogenesis is probably initiated by an injury to progenitor cells that affects their genetic structure.[6,7] Under normal circumstances, intrinsic cell-cycle mechanisms are activated to limit the proliferation of DNA-damaged cells. However, these mechanisms also may be affected by DNA damage. The *p53* tumor suppressor gene, which is located on the short arm of chromosome 17, is involved in cellular protection. p53 protein has a role in the arrest of cell growth to allow time for DNA repair and in the induction of cell death through activation of apoptosis.[8] Alterations in the *p53* gene can be studied by examination of the expression of p53 protein in fresh or archived tissue sections and smears using antibodies to various epitopes. Normally, p53 protein has a very short half-life, and it is usually not detected in BM using immunohistochemistry. The protein may be overexpressed as a result of a gene mutation,

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which is usually associated with protein stabilization, or as a result of insults, which lead to cell stress or DNA damage. However, several studies of patients with Fanconi anemia or RA have failed to detect mutations in the *p53* gene.[9–15] A similar study in SDS showed no *p53* gene mutations.[16]

In our previous study of a small number of patients, p53 protein was not overexpressed in acquired aplastic anemia, while it was commonly overexpressed in RA.[17] In the absence of previous reports of *p53* gene mutation, we hypothesized that *p53* gene alterations associated with p53 protein overexpression in these BM failure disorders may correlate with cell stress and DNA damage, and that diseases with closely similar risk values for acute leukemia may overexpress p53 protein with a comparable prevalence. To test this hypothesis, we studied p53 protein overexpression in patients with SDS and compared them with other disorders with variable risks for leukemia, such as RA, aplastic anemia, and miscellaneous acquired cytopenias.

MATERIALS AND METHODS

We studied BM biopsy sections from 46 patients with RA, 14 patients with aplastic anemia, 11 patients with various acquired cytopenias, and 9 patients with SDS. None of the patients with SDS was in the myelodysplastic syndrome phase, which was defined by having at least 2 lineages with significant dysplasia.[18] Patients with acquired cytopenia included the following: 2 with immune-mediated thrombocytopenia and 1 each with thrombotic thrombocytopenic purpura, systemic lupus erythematosus, orotic aciduria, osteomyelitis, chronic renal failure, inflammatory bowel disease, anemia of chronic disease, hypothyroidism, and liver disease with hypersplenism. Bone marrow biopsies from 37 hematologically normal individuals provided the controls. These individuals had BM examinations mostly for staging of lymphoproliferative disorders, and none showed involvement. Biopsies were fixed in formalin or B5 fixative and then decalcified in 10% nitric acid. Previous studies on BM biopsies have suggested the preservation of p53 protein with both formalin and B5 fixatives.[19–21] Four-micrometer sections were mounted on poly-L-lysine–coated slides. All slides were subjected to a heat-based antigen retrieval method using Target Retrieval Solution (Dako Corporation, Carpinteria, Calif) containing citrate buffer (pH 6) in a water bath (95°C–99°C) for approximately 30 minutes. Using avidin-biotin-peroxidase complexes (ABC method), BM biopsies were stained with anti–human p53 protein DO-7 monoclonal antibody (Dako). This sensitive antibody recognizes both wild and mutant types of p53.[22,23] All slides were stained using an autostainer (Dako). Sections of a p53-positive adenocarcinoma of the colon were used as the positive control. The presence of any cell with clear and unequivocal nuclear staining identified positive cases.[17,19] Cases positive for p53 were graded as follows: weak, less than 5% of all nucleated marrow cells; moderate, 5% to 30%; and strong, greater than 30%. Negative cases were graded as 0%. Biopsies were accepted when they had a minimum of 2000 nucleated cells. All slides were reviewed blindly without knowledge of the underlying disease. Cytogenetic analysis was performed for patients with adequate specimens. Twenty-four–hour unstimulated BM lymphocyte cultures were set up and harvested using standard procedures. The slides were G-banded using pancreatin-Giemsa method. A total of 20 metaphases were analyzed.

Statistical analyses were performed using Stata 7.0 (Stata Corp, College Station, Tex). The binomial probability test and 2-sided Fisher exact test were used when applicable.

RESULTS

The demographic data for patients in each disease category are shown in Table 1. The study included 9 patients with SDS, 37 hematologically normal controls, and 71 patients with acquired cytopenic hematologic diseases. The slight sex imbalances were not significant ($P > .2$ for all categories by binomial probability test). The median age of patients with SDS was 6 years, reflecting its early presentation and diagnosis. The patients with RA included 39 patients with primary RA and 7 patients with RA secondary to chemotherapy for breast cancer (4 patients), non-Hodgkin lymphoma (2 patients), and multiple myeloma (1 patient).

Table 1. Patient Data

Disease*	No. of Cases	No. of Males/ No. of Females	Median Age, y (Range)
RA	46	26/20	67 (1–92)
AA	14	9/5	35 (17–63)
SDS	9	4/5	6 (2.5–37)
AC	11	8/3	40 (1–62)
Control	37	22/15	44 (4–62)

* RA indicates refractory anemia; AA, acquired aplastic anemia; SDS, Shwachman-Diamond syndrome; and AC, miscellaneous acquired cytopenias (see "Materials and Methods").

Table 2. Results of p53 Protein Staining in Bone Marrow Biopsies

Disease*	No. of Cases Studied	No. Positive (%)†	No. Strong (%)‡	No. Moderate (%)‡	No. Weak (%)‡
RA	46	33 (72)	3 (9)	3 (9)	27 (82)
AA	14	0 (0)	0 (0)	0 (0)	0 (0)
SDS	9	7 (78)§	1 (14)	2 (29)	4 (57)
AC	11	0 (0)	0 (0)	0 (0)	0 (0)
Control	37	0 (0)	0 (0)	0 (0)	0 (0)

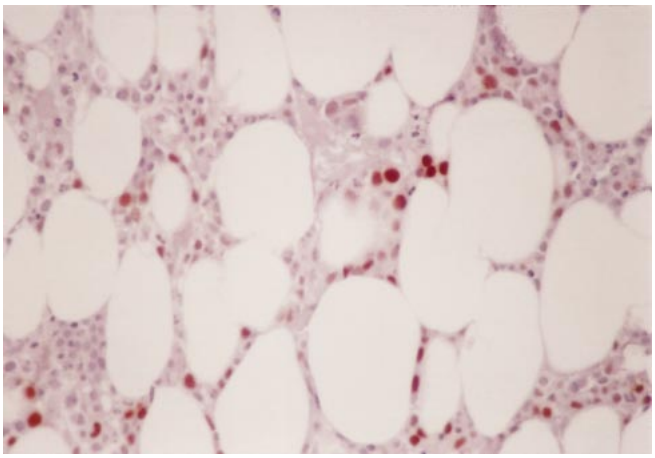
* RA indicates refractory anemia; AA, acquired aplastic anemia; SDS, Shwachman-Diamond syndrome; and AC, miscellaneous acquired cytopenias (see "Materials and Methods").

† Percentage of total cases that were positive.

‡ Percentage of positive cases according to the following grading score: weak, <5% of all nucleated marrow cells; moderate, 5% to 30%; and strong, >30%.

§ $P < .001$ for SDS compared to control, AA, or AC; $P = 1.0$ for SDS compared to RA.

p53 staining in BM biopsies was seen in RA and SDS, but there was no detectable staining in any of the aplastic anemia, acquired cytopenia, or control specimens. The results of immunohistochemical staining are summarized in Table 2. The prevalence of p53 BM overexpression in SDS is comparable to that seen in RA (78% vs 72%, $P = 1.0$ by 2-sided Fisher exact test). In most specimens in which there was expression of p53, the expression was weak. Thus, among the 33 patients with RA whose biopsies were positive for p53, 27 (82% of the positive group) reactions were weak. Among 7 patients with SDS whose specimens expressed p53, 4 (57% of the positive group) reactions were also weak. These results are not significantly different ($P = .3$ by 2-sided Fisher exact test). Within the RA group, 29 of the 39 patients with primary RA were p53 positive, while 4 of the 7 patients with secondary RA were p53 positive. There was no statistical difference in p53 staining between primary and secondary RA ($P = .3$ by a 2-sided Fisher exact test). Most positive cells have an immature morphology with round nuclei, fine chromatin pattern, and prominent nucleoli. Mature and maturing cells with identifiable morphology, such as band and segmented neutrophils, megakaryocytes, and nucleated red cells, were always negative.



Moderate expression of p53 protein in a bone marrow with refractory anemia (avidin-biotin-peroxidase immunostain, original magnification ×400).

Cytogenetic studies were available on 8 patients with SDS and 15 patients with RA. All patients with SDS showed normal results. Two patients with RA (13%) showed clonal cytogenetic abnormalities. One patient had monosomy 17, which was associated with strong p53 overexpression. This patient was alive and well for 1½ years of follow-up. The other patient had trisomy 8, which was associated with moderate overexpression of p53 (Figure). The patient died of heart failure 2½ years after diagnosis, and an autopsy showed no evidence of acute leukemia. All remaining 13 patients with RA with normal cytogenetics had either weak or no overexpression of p53. On the other hand, 2 RA patients (4%) developed acute myeloid leukemia. Both had weak overexpression of p53, and unfortunately both had no cytogenetic studies. Although the number of patients was small, higher expression of p53 in RA seemed to correlate with cytogenetic abnormalities rather than leukemic transformation. Yet, patients with SDS showed no such correlation with cytogenetic abnormalities given that all cytogenetic studies were normal.

COMMENT

p53 protein overexpression in BM is a valuable tool for studies of BM failure syndromes. p53 protein is not overexpressed in the BM of hematologically normal individuals.[17,19] Our preliminary study indicated the presence of weak p53 overexpression in patients with systemic infection and shortly following chemotherapy, but not in other BM conditions.[24] None of the patients in this study had these conditions. In a previous study in which we compared hypocellular RA with acquired aplastic anemia, we recommended the use of p53 protein overexpression to differentiate between these disorders since the BM of patients with aplastic anemia did not overexpress p53 protein.[17] In view of the literature reports in which *p53* gene mutations were not found in Fanconi anemia, SDS, and RA, p53 protein overexpression probably reflects a functional *p53* gene that has been up-regulated to provide additional protection for cells with damaged and potentially hazardous DNA. We confirmed this hypothesis in a follow-up study, which indicated overexpression of $p21^{waf-1}$, the downstream mediator of p53, in most cases overexpressing p53 protein.[25]

Using p53 protein overexpression as a potential marker for DNA genetic alterations, which is a crucial step in the initiation of the leukemogenic process, we compared the prevalence of p53 protein overexpression in SDS and RA among other diseases with various risks for leukemic transformation. Shwachman-Diamond syndrome and RA overexpressed p53 protein with a comparable prevalence (78% vs 72%, respectively). However, other acquired disorders, that is, aplastic anemia and acquired cytopenias, in addition to control individuals, did not exhibit overexpression of p53 protein. It is tempting to speculate that DNA in patients with SDS is as susceptible to damaging insults as that of RA patients, hence, the higher risk in both groups to develop acute leukemia (10% in RA and 5% in SDS).[2–4] This study confirms that p53 protein overexpression cannot be used to differentiate RA from an inherited BM failure disorder, such as SDS. However, this event may herald transformation. Long-term follow-up studies are needed to investigate the potential use of p53 protein staining in the BM as a predictor for the development of a leukemic process. Although SDS may be representative of other inherited BM failure syndromes with a leukemic propensity, such as Fanconi anemia, Diamond-Blackfan anemia, or dyskeratosis congenita, studies of each of these disorders are needed to investigate this hypothesis.

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